

Dual control of hydrogen cyanide biosynthesis by the global activator GacA in *Pseudomonas aeruginosa* PAO1

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Received 30 March 2001; received in revised form 19 April 2001; accepted 19 April 2001

First published online 11 May 2001

Abstract

The global response regulator GacA of *Pseudomonas aeruginosa* PAO1 positively controls the production of the quorum sensing signal molecule *N*-butanoyl-homoserine-lactone (C4-HSL) and hence the synthesis of several C4-HSL-dependent virulence factors, including hydrogen cyanide (HCN). This study presents evidence that GacA positively influences the transcription of the *rhII* gene, specifying C4-HSL synthase, explaining the quorum sensing-dependent transcriptional control of the HCN biosynthetic genes (*hcnABC*). In addition, GacA was found to modulate *hcn* gene expression positively at a post-transcriptional level involving the *hcnA* ribosome-binding site. Thus, the activating effect of GacA on cyanogenesis results from both transcriptional and post-transcriptional mechanisms. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: GacS/GacA two-component system; Secondary metabolism; Hydrogen cyanide; Quorum sensing; *Pseudomonas aeruginosa*

1. Introduction

Among bacteria that utilize quorum sensing regulation as part of their pathogenic lifestyle, the opportunistic pathogen *Pseudomonas aeruginosa* is perhaps the best understood example in terms of the mechanisms involved in the expression of pathogenicity [1,2]. Cell density-dependent regulation of *P. aeruginosa* is essentially mediated by the signal molecules *N*-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL; produced by the LasI enzyme) and *N*-butanoyl-homoserine lactone (C4-HSL; produced by the RhII enzyme). These signals interact with the transcriptional regulators LasR and RhIR, respectively, to activate the expression of genes encoding virulence factors, including hydrogen cyanide (HCN) [3,4]. The LasR/LasI and RhIR/RhII systems are organized in a regulatory cascade; a further effector, the *Pseudomonas* quinolone signal, modulates the expression of both systems [5].

C4-HSL production in *P. aeruginosa* is under positive control of the GacS/GacA system [6]. This two-component

system is conserved in many Gram-negative bacteria. GacS is a transmembrane sensor protein, which responds to an unknown signal by autophosphorylation; phosphotransfer from GacS to the amino-terminal domain of the response regulator GacA occurs subsequently. This mechanism has been shown recently for the GacS/GacA homologs BarA and UvrY in *Escherichia coli* [7]. The GacS/GacA system also regulates *N*-acyl-HSL synthesis in a biocontrol strain of *Pseudomonas aureofaciens* [8] and in the plant pathogen *Pseudomonas syringae* [9]. In *P. aeruginosa* a *gacA* mutant exhibits strongly decreased virulence in the nematode *Caenorhabditis elegans*, in the plant *Arabidopsis thaliana*, and in burnt mice [10–12]. The mechanisms by which GacA regulates C4-HSL synthesis and virulence are only partially understood; it has been shown that GacA has a positive effect on *lasR* and *rhIR* transcription [6]. Recently, Blumer et al. [13] have demonstrated that in *P. fluorescens* CHA0, a bacterium that does not produce *N*-acyl-HSL, GacA control manifests itself at the level of translation of target genes. This control involves a region close to the ribosome-binding site (RBS) of target genes such as the *hcnABC* structural genes for HCN synthase. The RBS region of the *P. fluorescens hcnA* gene might be recognized by a translational regulatory complex including the RNA-binding protein RsmA

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[13]. One function of GacA could be to alleviate RsmA-mediated translational repression of target genes, e.g. by inducing the synthesis of a regulatory RNA capable of sequestering RsmA [14,15]. However, the direct GacA targets are still elusive.

Here we report that in *P. aeruginosa*, GacA controls the expression of the *hcnABC* genes [16] at two levels: first, via a transcriptional activation of the *rhlI* gene and second, via a post-transcriptional mechanism involving the RBS region of the *hcnA* gene.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. aeruginosa strains used were the wild-type PAO1 and PAO6281 (*gacA*:: Ω Sp^R/Sm^R) [6]. Bacterial strains were routinely grown in nutrient yeast broth (NYB) or on nutrient agar plates at 37°C [6]. When required, tetracycline was added to the medium at a concentration of 25 μ g ml⁻¹

(*E. coli*) or 125 μ g ml⁻¹ (*P. aeruginosa*) and spectinomycin/streptomycin were added at a concentration of 1000 μ g ml⁻¹.

2.2. DNA manipulation and cloning procedures

DNA cloning and plasmid preparations were performed according to standard methods [17]. Plasmids pME3826 (carrying a translational *hcnA'*-*lacZ* fusion) and pME3850.1 (carrying a transcriptional *hcnA*-*lacZ* fusion), have been described [16]. The construction of the translational *rhlI'*-*lacZ* fusion on vector pME6010 [18], resulting in plasmid pME3846 (Fig. 1) will be described elsewhere. To generate plasmid pME3855 (Fig. 1), a 1.24-kb *Bam*HI/*Pst*I fragment from pME3840 [16] containing the *rhlI* promoter and almost the entire coding region of *rhlI* (lacking the last nine codons) was fused in frame with the *lacZ* reporter from pNM481 [19] carried by the vector pME6010 [18]. Plasmid pME3861 containing the *rhlI* gene under the control of the *tac* promoter (Fig. 1) was obtained as follows: primer R3M (5'-GCGCGGTA-

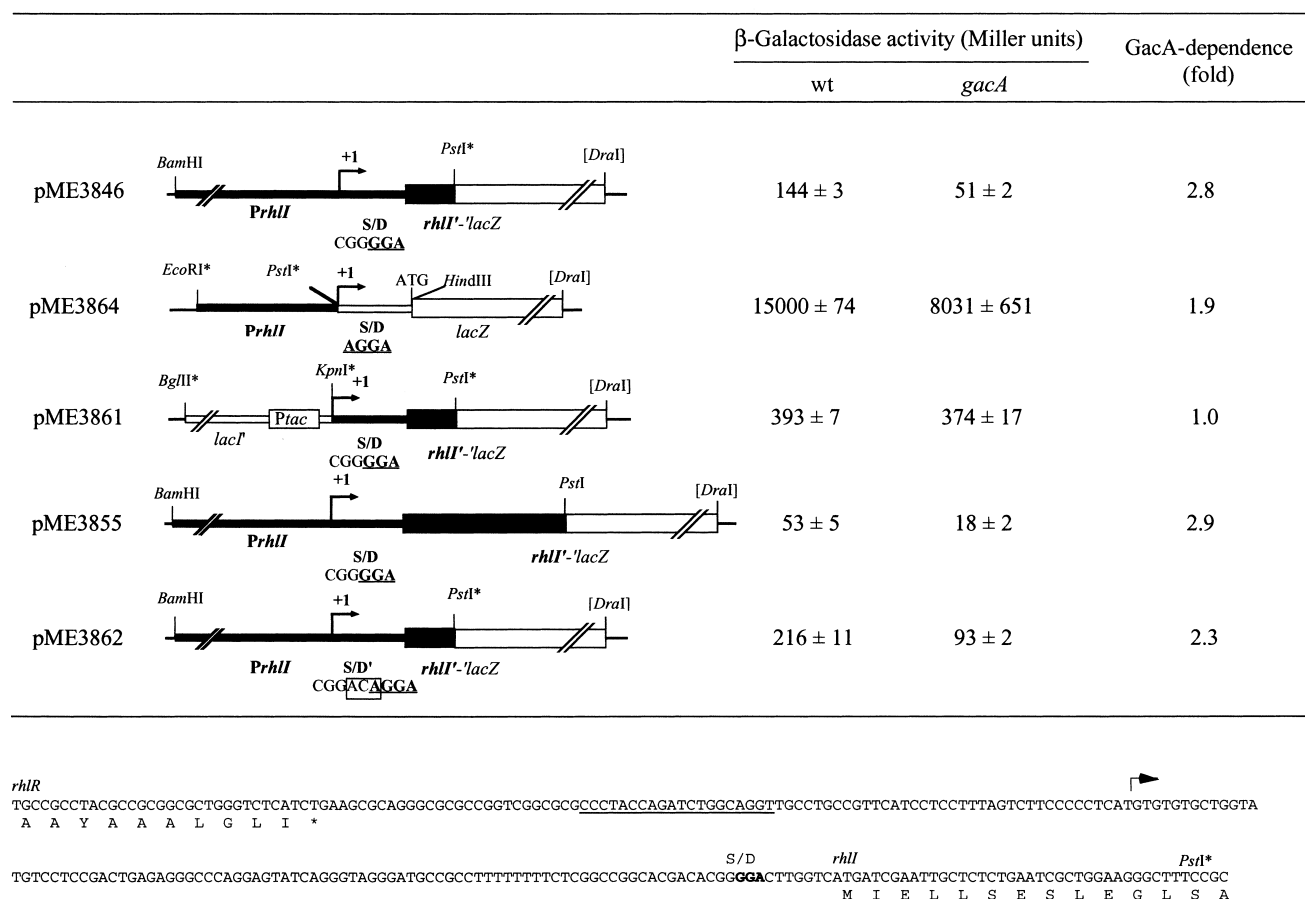


Fig. 1. Translational and transcriptional *lacZ* fusions used to study GacA control of *rhlI* expression. All constructs shown are derived from vector pME6010 [18]. The relevant *rhlI* sequence [24] is shown below. S/D, putative Shine Dalgarno sequence, indicated in bold; the *rhlI* transcriptional start site (K. Winzer, personal communication) is indicated by an arrow; a potential *lux* box (RhIR- and/or LasR-binding site) is underlined. *rhlI* sequences are shown by heavy black lines, *ptac* and *lacZ* by white boxes; artificially introduced restriction sites are marked with *. The 3-bp ACA insertion in pME3862 is boxed. β -Galactosidase activities were determined in *P. aeruginosa* PAO1 (wild-type=wt) and PAO6281 (*gacA*) grown in NYB to an OD₆₀₀ of 1.01.2. Data are the means \pm standard deviation of three measurements.

CCTGTGTGTGCTGGTAT-3'), which anneals to the region around the +1 transcription start site, and primer R2 (5'-AAAACTGCAGCGGAAAGCCCTTCCAGCG-3'), which is complementary to codons 9 to 14 of *rhII*, were used to PCR amplify the *rhII* leader region from pME3846. The resulting 141-bp fragment was cleaved with *KpnI* and *PstI* (artificial sites, underlined) and cloned into a pUK21 derivative containing the *tac* promoter [13]. A 1.14-kb *BglIII/PstI* fragment of this construct (containing the *tac* promoter) was then used to replace the 0.7-kb *BamHI/PstI* fragment of plasmid pME3846 (containing the native promoter), thereby creating plasmid pME3861. The insertion of three additional bases into the RBS of the translational *rhII'*-*lacZ* fusion (on pME3846) was generated by the overlap extension method [20] using primer MR1 (5'-GCCGGCACGACACGGACAGGACTTGGT-3') to introduce the mutation (underlined). Using pMP21 [21] as the template, two PCR products of 742 bp and 200 bp were generated with the primers R2 plus R1 (5'-GCTGGAGCGATACCAGATGCA-3'), which anneals 702 nt upstream of the *rhII* translational start site, and MR1 plus a primer (*lacZ*) annealing within the *lacZ* sequence (5'-TGCTGCAAGGCGATTAAGTGGG-3'), respectively. A mixture of these two PCR products was used as the template to amplify the *rhII* region containing the mutation in the RBS by the use of primers R1 and *lacZ*; the resulting product was cut with *BamHI* and *PstI* and inserted into pME3846, creating plasmid pME3862 (Fig. 1). The promoter probe vector pME6522 [13] containing a promoterless *lacZ* gene was used to construct a transcriptional *rhII-lacZ* fusion on plasmid pME3864 as follows. The *rhII* promoter region was amplified by PCR using the *EcoRI*-tagged primer

R4T (5'-GCTCGAATTCGATCCTCAACGGCCT-3'), which anneals 560 bp upstream from the +1 transcriptional start site, and the *PstI*-tagged primer R5T (5'-GCTCCTGCAGATGAGGGGGAAGACTAAAG-3'), which anneals to the +1 transcriptional start site (K. Winzer, personal communication). The 580-bp amplified region was digested with *EcoRI* and *PstI* and cloned into pME6522 [13]. In plasmid pME3843 [13] the *hcnA* promoter of plasmid pME3826 [16] has been replaced by the constitutively expressed *tac* promoter. The 76-bp *KpnI/PstI* fragment of pME3843 containing the *hcn* 5' untranslated region and the first nine codons of the *hcnA* coding region, was substituted by a synthetic *KpnI/PstI* linker carrying a mutated RBS (CACACAGG), resulting in plasmid pME3860.

2.3. β -Galactosidase assay

P. aeruginosa cells were grown at 37°C with shaking at 180 rpm in 50-ml flasks containing 20 ml NYB supplemented with 0.05% Triton X-100. The media were inoculated with 10^7 cells ml⁻¹. Cultures were sampled at different time points and assayed for β -galactosidase-specific activities according to the method of Miller [17].

3. Results

3.1. Regulation of *rhII* gene expression by GacA

To find out how the GacS/GacA system modulates C4-HSL production, we studied the expression of the *rhII* gene using transcriptional and translational *lacZ* fusions

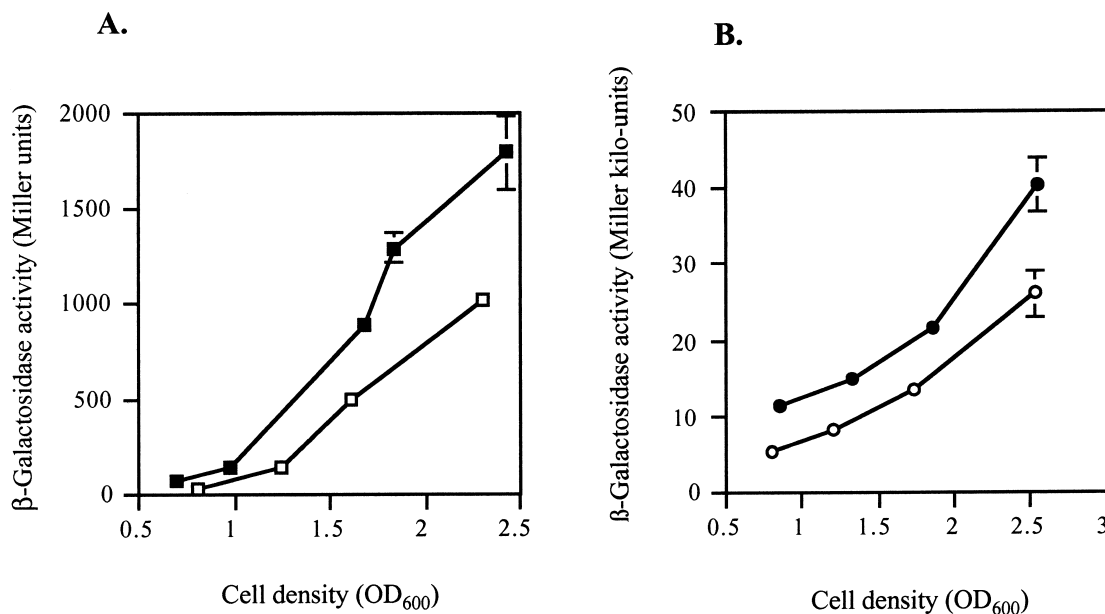


Fig. 2. Influence of GacA on *rhII* gene expression. β -Galactosidase activities resulting from (A) a translational *rhII'*-*lacZ* fusion on pME3846 (squares) and (B) a transcriptional *rhII-lacZ* fusion on pME3864 (circles) were determined in the wild-type *P. aeruginosa* PAO1 (filled symbols) and in the *gacA* mutant PAO6281 (open symbols) growing in NYB. Each value is the mean \pm standard deviation from three experiments.

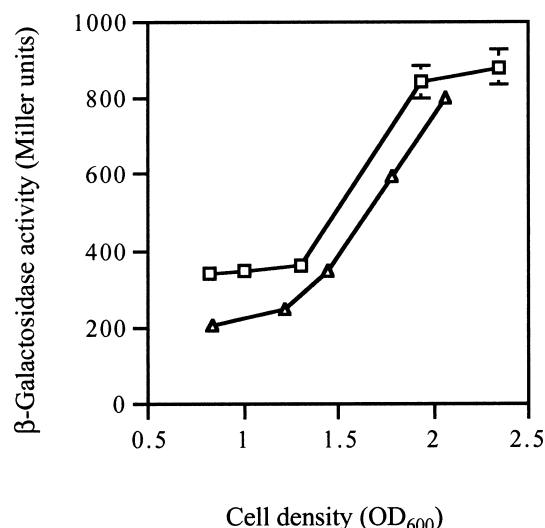


Fig. 3. Influence of GacA on *hcn* gene transcription. β -Galactosidase activities resulting from a transcriptional *hcn-lacZ* fusion on pME3850.1 was determined in the *P. aeruginosa* wild-type PAO1 (squares) and the *gacA* mutant PAO6281 (triangles). Each value is the mean \pm standard deviation from three experiments.

(carried by pME3846 and pME3864, respectively; see Fig. 1), in the wild-type strain PAO1 and the *gacA* mutant PAO6281. Both *lacZ* fusions were expressed progressively to high levels with increasing cell densities and introduction of a *gacA* mutation resulted in an approximately 2–3-fold reduction of expression throughout growth in rich medium (Fig. 2A,B). These data indicate that GacA essentially influences *rhII* expression at the transcriptional level.

Several controls were done. (i) When the *rhII* promoter was replaced by the *tac* promoter (*ptac*), as in pME3861, no GacA-dependent regulation was found, as expected (Fig. 1). (ii) A 3-bp (ACA) insertion in the RBS sequence

of *rhII*, in pME3862, had no effect on GacA control (Fig. 1). As will be shown below, a similar insertion into the RBS of *hcnA* abolishes post-transcriptional GacA control. (iii) A *rhII'*-*lacZ* translational fusion constructed near the 3' end of *rhII*, in pME3855, showed the same GacA dependence as a translational fusion made near the 5' end of *rhII* (in pME3846), indicating that the *rhII* coding sequence is irrelevant to GacA control.

The expression of the *rhII* gene is activated by RhlR in the presence of C4-HSL [22], presumably via the recognition of the *lux* box in the *rhII* promoter (Fig. 1). Since GacA positively controls *rhIR* transcription [6], the positive effect of GacA on *rhII* expression is likely to be mediated by RhlR.

3.2. *GacA* modulates *hcn* gene transcription essentially via the RhlR/C4-HSL system

The *hcn* promoter is activated by both the LasR/oxo-C12-HSL and the RhlR/C4-HSL systems in *P. aeruginosa* [16]. A *gacA* mutation has little influence on the amount of oxo-C12-HSL produced [6]. Using a translational *lasI'*-*lacZ* fusion, we compared *lasI* expression in the wild-type PAO1 and in the *gacA* mutant PAO6281. Expression was the same in both strains, within the experimental error (data not shown). Thus, GacA-dependent activation of *hcn* transcription is mediated essentially by the RhlR/C4-HSL system. To monitor this effect, we used a transcriptional *hcn-lacZ* fusion carried by plasmid pME3850.1, whose expression is solely dependent on quorum sensing [16]. As shown in Fig. 3, this construct was indeed expressed at lower levels in a *gacA* mutant than in the wild-type. However, although the difference was significant, it was smaller than that expected from previous measurements of the product, HCN [6]. Therefore, we con-

	Galactosidase activity (Miller units)		GacA-dependence (fold)
	wt	<i>gacA</i>	
<p>pME3826</p>	206 \pm 23	84 \pm 8	2.4
<p>pME3843</p>	7396 \pm 428	3607 \pm 203	2.1
<p>pME3860</p>	13192 \pm 428	17040 \pm 767	0.8

Fig. 4. Post-transcriptional, GacA-dependent regulation of *P. aeruginosa* translational *hcnA'*-*lacZ* fusions in *P. aeruginosa*. All constructs were made in the vector pME6010 as described in Section 2. The *hcn* region is indicated in boldface; the 3-bp ACA insertion in the *hcn* RBS of pME3860 is boxed; artificially introduced restriction sites are marked with *. β -Galactosidase activities were determined in *P. aeruginosa* PAO1 (wild-type=wt), and PAO6281 (*gacA*⁻) grown in NYB to an OD₆₀₀ of 1.0–1.2. Activities are the means \pm standard deviation from three measurements.

cluded that GacA exerted an additional control on *hcn* gene expression, i.e. downstream of transcription.

3.3. *GacA* additionally regulates *hcn* gene expression at a post-transcriptional level

In *P. fluorescens*, a 3-bp (ACA) insertion in the RBS region of the *hcnA* gene from this organism abolishes GacA control [13]. The untranslated *hcnA* leader sequences of *P. fluorescens* and *P. aeruginosa* are similar, albeit not identical [13]. Therefore, we tested in *P. aeruginosa* (i) whether a translational *hcnA'*–*lacZ* fusion would still be regulated by the GacS/GacA system after replacement of the native *hcn* promoter by the *tac* promoter and (ii) whether an analogous 3-bp insertion in the RBS region would interfere with GacA control. As shown by the *ptac* constructs pME3843 and pME3860 (Fig. 4), both assumptions proved correct. We conclude that the GacS/GacA system also has an impact on translation of the *hcnA* target gene in *P. aeruginosa*, via an unidentified pathway.

4. Discussion

In this study we have investigated an apparent dilemma. On the one hand, our previous observations have indicated that the global activator GacA positively influences the transcription of the *lasR* and *rhIR* genes as well as the production of C4-HSL and HCN [6] and that LasR and RhIR both activate the *hcn* promoter in *P. aeruginosa* [16]. On the other hand, we have reported that in *P. fluorescens* (an organism apparently devoid of *N*-acyl-HSLs), GacA control of *hcn* gene expression does not involve the *hcn* promoter, but only the RBS region [13,23]. We now show that in *P. aeruginosa* the GacS/GacA regulatory system has a dual role: this system upregulates the *hcn* promoter, essentially by activating the RhIR/C4-HSL part of the quorum sensing machinery, and also has a positive effect on *hcn* translation, via another, unknown mechanism. By analogy with the situation in *P. fluorescens* [13], this latter mechanism may involve the RNA-binding protein RsmA. Experiments to clarify this point are currently being done.

Chancey et al. [8] have observed a similar complex regulatory effect of the GacS/GacA system on the production of phenazine antibiotics in *P. aureofaciens*. In this bacterium, the GacS/GacA system controls transcription of the *N*-hexanoyl-homoserine lactone (C6-HSL) synthase gene *phzI*. However, the addition of exogenous C6-HSL does not complement a *gacA* or a *gacS* mutant of *P. aureofaciens* for phenazine production, indicating that the regulation of phenazine production by the GacS/GacA system also occurs downstream of PhzR/PhzI control, presumably at the level of the *phz* structural genes [8].

We note that the *rhII*–*lacZ* transcriptional fusion on pME3864 displayed a much higher activity than did the translational fusion pME3846 (Fig. 1). Furthermore, the

expression of the *rhII'*–*lacZ* translational fusion driven from the strong *tac* promoter on pME3861 was also very low (Fig. 1). It appears that translational expression of the *rhII* gene may be limited either by restricted translation initiation or by poor mRNA stability. Since a transcriptional *lacZ* fusion constructed within the *rhII* structural gene is expressed at high levels in *P. aeruginosa* PAO1 [25], it is more likely that the low expression of the translational *rhII'*–*lacZ* fusion results from poor translation initiation.

In conclusion, global control of secondary metabolism by the GacS/GacA system in *P. aeruginosa* does not operate via a simple, linear signal transduction pathway, but involves at least two layers of regulation. In the first, quorum sensing is modulated. In the second, which depends on the RBS of target gene, *N*-acyl-HSLs are probably not required.

Acknowledgements

We thank Cornelia Reimann for discussion and Klaus Winzer for communication of unpublished results. This work was supported by the Swiss National Foundation for Scientific Research (31-56608.99) and the European Biotechnology project BIO4CT960119.

References

- [1] Rumbaugh, K.P., Griswold, J.A., Iglewski, B.H. and Hamood, A.N. (1999) Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infect. Immun.* 67, 5854–5862.
- [2] Pearson, J.P., Feldman, M., Iglewski, B.H. and Prince, A. (2000) *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. *Infect. Immun.* 68, 4331–4334.
- [3] Pearson, J.P., Passador, L., Iglewski, B.H. and Greenberg, E.P. (1995) A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 92, 1490–1494.
- [4] Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., Bally, M., Chapon, V., Salmond, G.P., Bycroft, B.W., Lazdunski, A., Stewart, G.S.A.B. and Williams, P. (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 92, 9427–9431.
- [5] McKnight, S.L., Iglewski, B.H. and Pesci, E.C. (2000) The *Pseudomonas* quinolone signal regulates *rhI* quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182, 2702–2708.
- [6] Reimann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. and Haas, D. (1997) The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol. Microbiol.* 24, 309–319.
- [7] Pernestig, A.K., Melefors, O. and Georgellis, D. (2001) Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. *J. Biol. Chem.* 276, 225–231.
- [8] Chancey, S.T., Wood, D.W. and Pierson, L.S. (1999) Two-component transcriptional regulation of *N*-acyl-homoserine lactone produc-

- tion in *Pseudomonas aureofaciens*. Appl. Environ. Microbiol. 65, 2294–2299.
- [9] Kitten, T., Kinscherf, T.G., McEvoy, J.L. and Willis, D.K. (1998) A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. 28, 917–929.
- [10] Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G. and Ausubel, F.M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. Science 268, 1899–1902.
- [11] Rahme, L.G., Tan, M.W., Le, L., Wong, S.M., Tompkins, R.G., Calderwood, S.B. and Ausubel, F.M. (1997) Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA 94, 13245–13250.
- [12] Tan, M.W., Mahajan-Miklos, S. and Ausubel, F.M. (1999) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc. Natl. Acad. Sci. USA 96, 715–720.
- [13] Blumer, C., Heeb, S., Pessi, G. and Haas, D. (1999) Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. USA 96, 14073–14078.
- [14] Romeo, T. (1998) Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol. Microbiol. 29, 1321–1330.
- [15] Aarons, S., Abbas, A., Adams, C., Fenton, A. and O’Gara, F. (2000) A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. J. Bacteriol. 182, 3913–3919.
- [16] Pessi, G. and Haas, D. (2000) Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhIR in *Pseudomonas aeruginosa*. J. Bacteriol. 182, 6940–6949.
- [17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [18] Heeb, S., Itoh, Y., Nishijyo, T., Schnider, U., Keel, C., Wade, J., Walsh, U., O’Gara, F. and Haas, D. (2000) Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. Mol. PlantMicrobe Interact. 13, 232–237.
- [19] Minton, N.P. (1984) Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene 31, 269–273.
- [20] Mikaelian, I. and Sergeant, A. (1996) Modification of the overlap extension method for extensive mutagenesis on the same template. In: Methods in Molecular Biology, Vol. 57, pp. 193–202. Humana, Totowa, NJ.
- [21] Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S., Lazdunski, A. and Williams, P. (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol. Microbiol. 17, 333–343.
- [22] Latifi, A., Foglino, M., Tanaka, K., Williams, P. and Lazdunski, A. (1996) A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21, 1137–1146.
- [23] Blumer, C. and Haas, D. (2000) Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. Microbiology 146, 2417–2429.
- [24] Ochsner, U.A. and Reiser, J. (1995) Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 92, 6424–6428.
- [25] Van Delden, C., Pesci, E.C., Pearson, J.P. and Iglewski, B.H. (1998) Starvation selection restores elastase and rhamnolipid production in a *Pseudomonas aeruginosa* quorum-sensing mutant. Infect. Immun. 66, 4499–4502.